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Electrical activity-dependent regulation of the acetylcholine receptor δ-subunit gene, MyoD, and myogenin in primary myotubes

(gene regulation/neuromuscular synapse/skeletal muscle/denervation)

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ABSTRACT **Expression of the skeletal muscle acetylcholine receptor (AChR) is regulated by nerve-evoked muscle activity. Studies using transgenic mice have shown that this regulation is controlled largely by transcriptional mechanisms because responsiveness to electrical activity can be conferred by transgenes containing cis-acting sequences from the AChR subunit genes. The lack of a convenient muscle cell culture system for studying electrical activity-dependent gene regulation, however, has made it difficult to identify the important cis-acting sequences and to characterize an electrical activity-dependent signaling pathway. We developed a muscle culture system to study the mechanisms of electrical activity-dependent gene expression. Gene fusions between the murine AChR δ-subunit gene and the human growth hormone gene were transfected into primary myoblasts, and the amount of growth hormone secreted into the culture medium from either spontaneously electrically active or inactive myotube cultures was measured. We show that 181 bp of 5′-flanking DNA from the AChR δ-subunit gene are sufficient to confer electrical activity-dependent gene expression. In addition, we show that the rate of AChR δ-subunit gene expression differs among individual nuclei in a single myotube but that highly expressing nuclei are not necessarily colocalized with AChR clusters. We also show that expression of MyoD and myogenin are regulated by electrical activity in primary myotube cultures and that all nuclei within a myotube express similar levels of MyoD and similar levels of myogenin.**

The skeletal muscle acetylcholine receptor (AChR) is a heteropentamer composed of four subunits (α_2, β, γ or ε, δ) that are encoded by separate genes, which are regulated similarly during development (1). AChR mRNA is expressed at high levels throughout developing muscle, but expression decreases as the myofibers are innervated, and in innervated adult muscle, AChR mRNA is largely restricted to the single synaptic site (2–7). Expression of AChR mRNA increases after denervation, and this additional AChR mRNA is found throughout the myofiber (4, 5, 8). This increase in mRNA is prevented by direct stimulation of denervated muscle (9), indicating that loss of electrical activity rather than loss of putative nerve-derived trophic factors is largely responsible for the increase in AChR expression after denervation. Studies using transgenic mice have shown that electrical activity regulates AChR gene expression by transcriptional mechanisms and that cis-acting elements that confer electrical activity-dependent gene expression are contained in <1 or 2 kbp of 5′-flanking DNA from the α- and δ-subunit genes, respectively (7, 10).

Little is known about the pathway that couples changes in muscle cell electrical activity to changes in AChR gene expression. Although transcriptional mechanisms are important (7, 10, 11), transgenic mice are neither ideal for delineating the critical electrical activity-dependent regulatory sequences nor for characterizing an electrical activity-dependent signaling pathway. For these reasons, we developed a primary rat muscle cell culture system to investigate how muscle cell electrical activity regulates AChR gene expression. We show here that 181 bp of 5′-flanking DNA from the AChR δ-subunit gene, which confers myotube-specific gene expression (12) and includes an enhancer and a single E-box, contains cis-acting elements that are sufficient for electrical activity-dependent gene regulation.

MATERIALS AND METHODS

Cell Culture. Primary rat (13) or transgenic mouse (14) muscle cell cultures were prepared as described previously. Muscle tissue from fetal rats or from degenerating adult tibialis anterior muscle was dissociated, and cells were plated on Matrigel (Collaborative Research)-coated dishes (15). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum/10% horse serum. After transfection, cells were placed in differentiation medium (DMEM/10% horse serum) to induce myoblast fusion.

Gene Fusions. Gene fusions between the mouse AChR δ-subunit gene and the human growth hormone (hGH) gene were constructed as described (7, 12). Deletion of nt −500 to −182 placed activating sequences in the plasmid DNA closer to the δ-subunit promoter and increased expression in all cell types (A.M.S. and S.J.B., unpublished work). Because increased expression in myoblasts and fibroblasts obscured electrical activity-dependent regulation in myotubes, we inserted 2 kb of DNA (*Torpedo* dystrophin cDNA) (16) into the AChR δ-(−181/+25)-hGH plasmid upstream of nt −181 to minimize the effect of the activating plasmid sequences. The rabbit muscle creatine kinase (CK) gene (−1.2 kbp/+54) (17) was cloned into the p0hGH vector (18). Myoblasts were transfected by using calcium phosphate precipitation.

hGH, Chloramphenicol Acetyltransferase (CAT), and RNase-Protection Assays. hGH radioimmunoassays were done according to the manufacturer's directions (Nichols Institute, San Juan Capistrano, CA). CAT assays and RNase protection assays were done as described (12). The rat δ subunit (19) and the skeletal muscle α-actin RNA (20) probes extended from nt +361 to +900 and from nt +650 to +1257, respectively. Total RNA was isolated by using guanidinium thiocyanate (21). To control for potential variability in the number of myotubes in electrically inactive and active cultures, we normalized the amount of AChR δ mRNA to α-actin mRNA, which does not change significantly after denervation (7).

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Abbreviations: hGH, human growth hormone; AChR, acetylcholine receptor; CAT, chloramphenicol transferase; CK, creatine kinase.

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Immunofluorescence. Cells were fixed with 2% (wt/vol) paraformaldehyde (in phosphate-buffered saline) and permeabilized with 0.5% Triton X-100 (in phosphate-buffered saline). AChRs were labeled with tetramethylrhodamine-conjugated α-bungarotoxin as described (22). hGH, MyoD, and myogenin were detected by indirect immunofluorescence with antibodies to hGH (7), MyoD (α-160–307, ref. 23) or myogenin (24) and with appropriate fluorochrome-conjugated secondary antibodies.

RESULTS

AChR δ-Subunit Gene Is Regulated by Electrical Activity in Primary Myotubes. We developed a primary rat muscle cell culture system to identify cis-acting elements in the AChR δ-subunit gene that are sufficient to confer electrical activity-dependent gene expression. We transfected primary rat myoblasts with AChR δ–hGH gene fusions and measured the amount of hGH secreted in culture medium, which was changed daily. Multinucleated myotubes form and begin to contract spontaneously 1 to 2 days after transfection. Tetrodotoxin (0.5 μg/ml), a sodium channel blocker, was added at the first sign of contraction to inhibit spontaneous electrical activity. Thus, we could measure the amount of hGH secreted from electrically active and inactive myotube cultures.

Because AChR genes are expressed immediately after myoblast fusion and before the onset of electrical activity, we reasoned that relatively stable reporter gene products might preclude detection of subsequent down-regulation of the AChR δ-subunit gene. Therefore, it was important to use a reporter gene product that would not accumulate in myotubes before the onset of electrical activity. Because hGH is secreted into the culture medium, which was changed and sampled daily, the amount of secreted hGH provided an accurate measure of the rate of δ-subunit transcription.

To control for variability in transfection efficiency and in the number of myotubes in each culture dish, we cotransfected cells with a gene fusion between the CAT gene and the myosin light chain 1 gene promoter, which is expressed specifically in myotubes (25) but is not regulated by electrical activity (10).

The amount of hGH secreted from myoblasts transfected with the AChR δ-(−1823/+25)–hGH gene fusion is low and increases 20- to 40-fold as myoblasts differentiate into myotubes (Fig. 1). Myotubes begin to contract spontaneously shortly thereafter, and they develop striations and often contain peripherally located nuclei; myotubes that were treated with tetrodotoxin are electrically inactive and lack these morphological specializations (see Fig. 3). Inactive myotubes secrete ≈3-fold more hGH than active myotubes (Fig. 1). Although expression from active and inactive myotubes decreases with time, inactive myotubes continue to secrete ≈3-fold more hGH than active myotubes (Fig. 1). These results indicate that the rate of AChR δ-subunit transcription is greater in electrically inactive myotubes than in active myotubes and that cis-acting elements in the AChR δ-subunit gene confer electrical activity-dependent expression of hGH in primary rat myotube cultures.

To control for the possibility that the difference in the amount of hGH secreted by active and inactive myotubes might be due to differences in the properties of secretion between electrically active and inactive myotubes, we transfected myoblast cultures with a gene fusion between hGH and the CK gene, which is expressed specifically in myotubes (26) but is not regulated by electrical activity (27). We found that active and inactive myotubes transfected with the CK–hGH gene fusion secrete similar amounts of hGH (Fig. 1 and Table 1). Thus, the difference in the amount of hGH secreted between electrically active and inactive myotubes transfected with the AChR δ–hGH gene fusion is due to regulatory sequences in the δ-subunit gene.

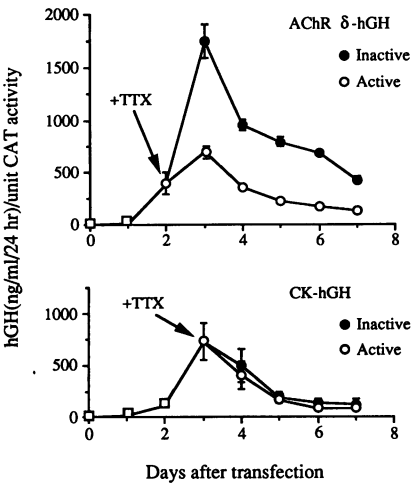

FIG. 1. AChR δ-subunit gene is regulated by electrical activity in primary myotubes. The amount of hGH secreted from the same culture was measured on 8 consecutive days, and the mean ± SEM for four sister cultures is shown. Electrically inactive myotubes transfected with a δ-(−1823/+25)–hGH gene fusion secrete ≈3-fold more hGH than active myotubes transfected with the same gene fusion. Electrically active and inactive myotubes transfected with a CK–hGH gene fusion secrete similar amounts of hGH. Loss of transfected DNA and/or cell loss is probably responsible for the progressive decrease in hGH expression seen in cultures transfected with either gene fusion; nevertheless, inactive myotubes transfected with δ-(−1823/+25)–hGH secrete ≈3-fold more hGH than active myotubes at all times. Squares indicate expression from myoblasts and myotubes before twitching was observed; tetrodotoxin (TTX) was added at the first sign of contraction.

181 bp of 5′-Flanking DNA Confer Electrical Activity-Dependent Gene Regulation. To determine the minimal sequence sufficient to confer electrical activity-dependent expression, we prepared 5′ deletions of the δ-subunit gene and transfected myoblasts with these additional gene fusions. Electrically inactive myotubes transfected with constructs containing 843 bp, 500 bp, or 181 bp of 5′-flanking DNA from the δ-subunit gene secrete ≈3-fold more hGH than electrically active myotubes (Table 1). These results demonstrate

Table 1. 181 bp of 5′-flanking DNA from the AChR δ-subunit gene contain cis-acting elements for electrical activity-dependent regulation

Gene fusion	Gene expression, inactive myotubes/ active myotubes	*n*
δ-(−1823/+25)–hGH	3.22 ± 0.56	11
δ-(−843/+25)–hGH	3.04 ± 0.20	8
δ-(−500/+25)–hGH	3.31 ± 0.56	5
δ-(−181/+25)–hGH	2.89 ± 0.29	4
CK-(−1.2 kbp/+54)–hGH	1.15 ± 0.06*	15

Myoblasts were cotransfected with hGH and myosin light chain–CAT gene fusions, and expression (hGH/CAT activity) in inactive myotubes was compared to expression in active myotubes. hGH expression is ≈3-fold greater in electrically inactive than in active myotubes transfected with AChR δ–hGH gene fusions. In contrast, electrically inactive and active myotubes transfected with a CK–hGH gene fusion secrete similar amounts of hGH. Thus, the increased hGH expression in electrically inactive myotubes transfected with the AChR δ–hGH gene fusions is due to electrical activity-dependent regulatory sequences contained within the δ-subunit gene. The means ± SEMs are shown.

*Significantly different from AChR δ-(−1823/+25)–hGH (Student's *t* test, $P = 0.01$).

that 181 bp of 5′-flanking DNA from the AChR δ-subunit gene are sufficient to confer electrical activity-dependent expression.

Endogenous and Transfected δ-Subunit Genes Are Regulated Similarly. Mice that carry an AChR δ-(−1823/+25)–hGH transgene express ≈15-fold more hGH in denervated than in innervated muscle (7). Similarly, denervated muscle contains ≈15-fold more AChR δ-subunit mRNA than innervated muscle (7). To determine whether the transfected AChR δ-subunit gene fusions and the endogenous AChR δ-subunit gene are regulated similarly in primary rat myotubes, we measured the level of endogenous AChR δ-subunit mRNA in active and inactive myotube cultures (*Materials and Methods*). We found that electrically inactive myotubes express 3.45 ± 0.22-fold more endogenous AChR δ-subunit mRNA than active myotubes ($n = 6$). Thus, the endogenous AChR δ-subunit gene and the transfected gene fusions are regulated similarly by electrical activity in primary rat myotube cultures. These results indicate that the difference in the level of δ-subunit mRNA in active and inactive myotubes is regulated largely by transcriptional mechanisms.

Heterogeneity of AChR δ-Subunit Gene Expression in Individual Myotubes. Because hGH is processed in the Golgi apparatus before secretion and because the Golgi apparatus is closely associated with nuclei, the nuclear source of hGH transcription can be determined by studying the intracellular distribution of hGH with immunocytochemical techniques (7). Previous studies using transgenic mice carrying an AChR δ-(−1823/+25)–hGH gene fusion showed that hGH expression is restricted to nuclei positioned near AChR clusters (7). Because AChR clusters are found on the surface of myotubes grown in cell culture, we wondered whether nuclei near these spontaneous AChR clusters preferentially express the AChR δ-subunit gene. Myotube cultures, prepared from transgenic mice satellite cells (7, 14), were stained with antibodies to hGH and with tetramethylrhodamine-conjugated α-bungarotoxin to determine whether nuclei near AChR clusters preferentially express hGH. We found that the abundance of hGH is, indeed, greater around a subset of nuclei in primary myotubes (Fig. 2). There is a poor correlation, however, between the position of highly expressing nuclei and the site of AChR clusters (Fig. 2); importantly, AChR clusters are not necessarily found near highly expressing nuclei, and highly expressing nuclei are not necessarily found near AChR clusters (Fig. 2). These results indicate that the presence of an AChR cluster is not sufficient to induce increased AChR δ-subunit transcription in nearby nuclei and that increased AChR δ-subunit expression is not sufficient to specify the site of an AChR cluster in primary rat myotubes.

Expression of MyoD and Myogenin Are Regulated by Electrical Activity in Primary Myotubes. To determine whether this culture system might be suitable for studying other gene products that are regulated by electrical activity, we studied the expression of MyoD and myogenin in active and inactive myotube cultures. MyoD and myogenin, basic helix–loop–helix proteins, are of particular interest because they are potential regulators of AChR subunit genes (28, 29), and after denervation, their mRNAs increase in abundance prior to AChR mRNA (30–32). We stained electrically active and inactive myotubes with antibodies to hGH, MyoD, and myogenin. As expected, we found that intracellular hGH expression is greater in electrically inactive than in active myotubes (Fig. 3). Like hGH, MyoD and myogenin are also more abundant in electrically inactive than in active myotubes (Fig. 3). Thus, the level of MyoD and myogenin protein expression is regulated by electrical activity in myotubes grown in cell culture.

In contrast to the nonuniform expression of the AChR δ-subunit gene (Fig. 2), all nuclei within a myotube appear to express similar levels of MyoD and similar levels of myogenin (Fig. 3). Thus, different nuclei within a multinucleated myotube express similar levels of MyoD and similar levels of myogenin protein, but they can express the AChR δ-subunit gene at different rates. These results indicate that additional

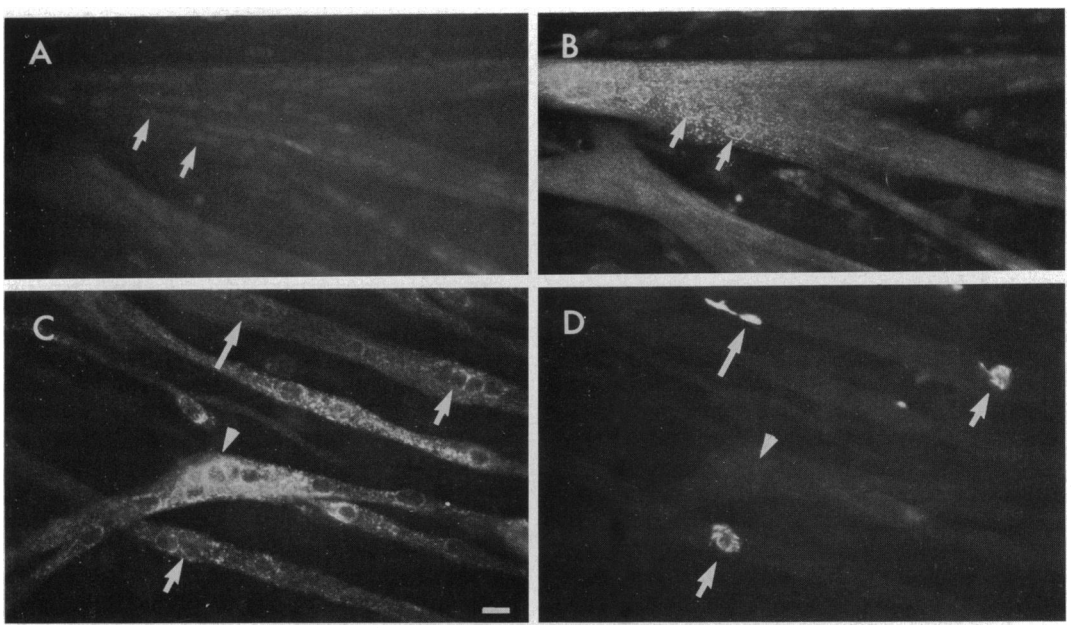

FIG. 2. AChR δ-subunit gene is expressed preferentially by a subset of nuclei in individual myotubes. Myotube cultures, prepared from satellite cells derived from transgenic mice carrying an AChR δ-(−1823/+25)–hGH gene fusion, were stained with antibodies to hGH and myogenin or with antibodies to hGH and tetramethylrhodamine-conjugated α-bungarotoxin. Different nuclei in an individual myotube express similar levels of myogenin (*A*), whereas hGH expression (*B*) is enhanced near a subset of nuclei (arrows) in the same myotube. The position of highly expressing nuclei (*C*), however, is not necessarily correlated with sites of AChR clusters (*D*). Importantly, AChR clusters are not always associated with highly expressing nuclei (arrowhead), and highly expressing nuclei are not always associated with AChR clusters (long arrow). Because a substantial fraction of the myotube contains highly expressing nuclei (*B* and *C*), it is not clear whether codistribution of AChR clusters (short arrows in *D*) and highly expressing nuclei (short arrows in *C*) is significant or occurs by chance. (Bar = 10 μm.)

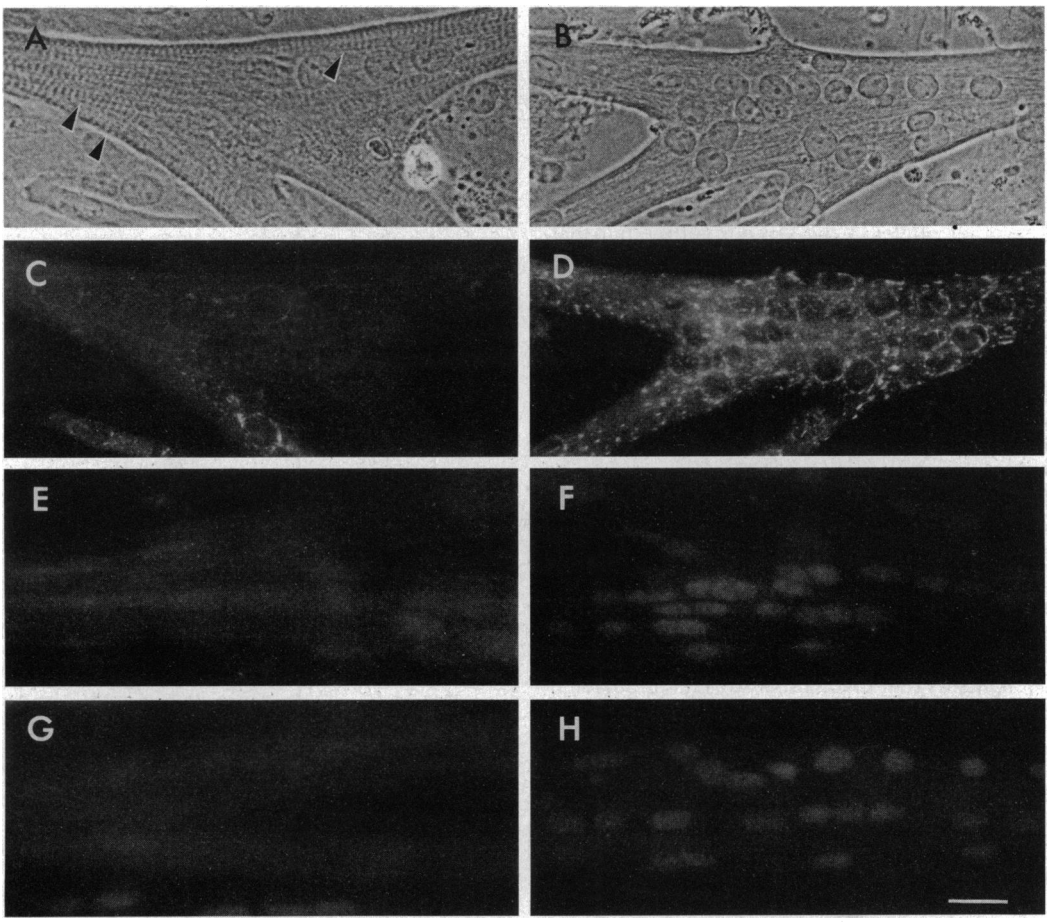

FIG. 3. MyoD and myogenin are regulated by electrical activity in primary myotubes. Electrically active and inactive transgenic mouse or primary rat myotubes were stained with antibodies to hGH, MyoD, or myogenin. Active myotubes (*A*) are morphologically distinguished from inactive myotubes (*B*) because active myotubes develop striations (arrows) and often have peripherally located nuclei. hGH expression is greater in electrically inactive (*D*) than active (*C*) myotubes. Similarly, expression of MyoD (*E* and *F*) and myogenin (*G* and *H*) is greater in electrically inactive (*F* and *H*) than active (*E* and *G*) myotubes. (Bar = 10 μm.)

factors, including potential regulation of MyoD or myogenin activities, control AChR δ-subunit gene expression.

DISCUSSION

We developed a primary rat muscle culture system to study electrical activity-dependent gene expression, and we show here that 181 bp of 5′-flanking DNA from the murine AChR δ-subunit gene are sufficient to confer electrical activity-dependent gene expression in primary rat myotubes. This is a convenient system for studying electrical activity-dependent gene expression for the following reasons: (*i*) an electrical activity-dependent signaling pathway can be characterized more readily in cultured myotubes than in animals, (*ii*) neither innervation nor extracellular stimulating electrodes are required because these primary myotubes contract spontaneously, and (*iii*) a decrease in the rate of transcription is rapidly and accurately reflected by a decrease in hGH secretion. In addition, because hGH expression can be visualized by immunofluorescence, this system may be useful for studying synapse-specific transcription.

The magnitude of electrical activity-dependent regulation that we detect in cell culture is less than we find in transgenic mice (7). One explanation for this discrepancy is that the frequency of electrical activity, which is known to influence AChR expression (33), may not be optimal in our cultures for repressing AChR expression. Further, because some myotubes (≈25%) in active cultures do not contract, expression from these inactive myotubes is likely to decrease the measured effect of electrical activity.

We find that spontaneously active myotubes regulate transfected and endogenous genes similarly, and these results indicate that regulation of δ-subunit expression by electrical activity is controlled largely by transcriptional mechanisms. Chahine *et al.* (27) have reported that 102 bp of 5′-flanking DNA from the rat AChR δ-subunit gene are sufficient to confer electrical activity-dependent regulation in cultured myotubes that are stimulated with extracellular electrodes; these myotubes, however, regulate the transfected δ-subunit gene fusion less strongly (4-fold) than the endogenous δ-subunit gene (10-fold). Although the reported difference in regulation between endogenous and transfected genes might suggest additional posttranscriptional regulation of AChR expression, it is possible that accumulation of a stable reporter product (luciferase) may account for the reported difference.

The mouse and rat δ-subunit sequences that confer electrical activity-dependent expression are 96% identical and contain a single E-box near the transcription start site (12, 27). Thus, comparison of these two sequences provides little additional information regarding the key cis-acting elements and indicates the need for further functional studies.

The AChR δ-subunit gene in adult myofibers is expressed preferentially in nuclei positioned near synaptic AChR clusters and in nuclei near AChR clusters in nonsynaptic regions of denervated muscle (7). These results raise the possibility that the position of highly expressing nuclei and clustering of

AChR protein are causally linked. Previous studies have shown that AChR mRNAs are expressed preferentially in a subset of nuclei in cultured myotubes (5, 34, 35); however, because it is difficult to combine *in situ* hybridization with immunocytochemistry, it has not been possible to determine whether these highly expressing nuclei are associated with clusters of AChR protein. We show that a subset of nuclei in cultured myotubes expresses the AChR δ-subunit gene at a greater rate than other nuclei in the same myotube but that the position of these highly expressing nuclei is not necessarily correlated with the location of AChR clusters. The presence of nuclei that express the δ-subunit gene at a high rate would not specify the site of an AChR cluster if these nuclei do not also express the other AChR subunit genes at a high rate; further studies will be necessary to determine whether all four AChR subunit genes are highly expressed in the same subset of nuclei. Moreover, AChR clusters in embryonic myotubes usually occur near patches of extracellular matrix that may contain signals important for AChR clustering (36, 37), and it is possible that highly expressing nuclei in primary cultures are not located near patches of such extracellular matrix. We do not know how transcriptional differences between nuclei in primary myotubes are established; nuclei in the synaptic region of adult muscle are also transcriptionally distinct from other myofiber nuclei (7, 38, 39), and mechanisms that distinguish subsets of nuclei in cultured myotubes could be similar to mechanisms that act to selectively regulate gene expression in synaptic nuclei. Our results indicate that the formation of AChR clusters at synaptic sites is not simply a consequence of the proximity of highly expressing nuclei; rather, it appears more likely that different signals, one that activates an AChR clustering pathway and a second that activates an AChR transcriptional pathway, coexist at synaptic sites.

There is evidence that an increase in intracellular [Ca^{2+}] and stimulation of protein kinase C activity may be involved in electrical activity-dependent inactivation of the chicken α-subunit gene (40–42). It is not clear how activation of protein kinase C might lead to inactivation of AChR subunit genes, but it is possible that this pathway involves posttranslational modification of myogenic basic helix–loop–helix proteins, resulting in their inability to activate their own genes or downstream genes, including the AChR subunit genes. A single binding site (E-box) for myogenic transcription factors, which is located close (−23/−15) to the transcription start site, is contained in the 181 bp of the AChR δ-subunit gene that confers electrical activity-dependent expression (12). This E-box and an enhancer, which is located between −150 and −50 and is active in all cell types tested, are critical for maximal expression of the AChR δ-subunit gene in myotubes (A.M.S. and S.J.B., unpublished work). Thus, it will be important to determine whether these elements are also required for electrical activity-dependent regulation. Moreover, it will be important to determine whether myogenic basic helix–loop–helix proteins are modified by electrical activity, and if so, whether such modification(s) are critical for regulation of AChR genes.

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